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Escherichia coli O157:H7 in a Cohort of Weaned, Preconditioned Range Beef Calves

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ABSTRACT

Escherichia coli O157:H7 (EC O157) is an important cause of foodborne disease. Cattle are reservoirs for the bacteria and are implicated in transmission to humans. Prevalence data in prefeedlot calves are limited. With the use of sensitive methods, a cohort of weaned beef calves ($n = 408$) was sampled before and after preconditioning to estimate fecal point prevalence and describe changes in EC O157 fecal shedding. EC O157 isolates were confirmed and characterized by PCR and pulsed-field gel electrophoresis. Calves from 29 cow-calf farms were commingled at three preconditioning sites and placed on a transition ration containing oxytetracycline (200 g/ton) for 45 days. Initial animal-level fecal point prevalence was 2.5% (95% confidence interval, 1 to 5) with a herd-level prevalence of 17.2% (95% confidence interval, 6 to 36). Point prevalence following the preconditioning feeding period was 0%. An unexpected finding in our study was EC O157 isolates that were Shiga toxin–deficient. Pulsed-field gel electrophoresis subtypes of EC O157 were unique in epidemiologically unlinked herds, except one herd that had two unique subtypes. We expected, but observed, neither increased fecal shedding in the cohort nor horizontal transmission of unique EC O157 subtypes. The absence of fecal shedding following the 45-day feeding period might be attributable to seasonal influences, inhibitory concentrations of oxytetracycline in the transition ration, or transient colonization that ended before sampling. EC O157 is apparently widely dispersed at low prevalence in U.S. prefeedlot, weaned calves.

Escherichia coli O157:H7 (EC O157) is an important cause of foodborne human disease. Complications related to infection include diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome *(17, 31)*. Ground beef has been identified as a vehicle since the earliest outbreaks of EC O157 and continues to be a significant vehicle *(1, 7, 36)*. Feedlot cattle supply a large amount of ground beef to U.S. markets *(46)*.

Control or reduction of EC O157 in beef cattle is important from a public health standpoint, as well as the perspective of livestock producers, slaughter houses, meat packers, and retailers. Not only does contaminated ground beef cause human illness and associated economic costs, detection of EC O157 in ground beef causes large economic losses for processors and distributors. A 1997 outbreak prompted a national recall of 25 million pounds of ground beef *(13)*. Recently, a large voluntary recall based on positive tests for EC O157 in lots of hamburger was expanded after human cases were associated with the contaminated ground beef. In total, 19 million pounds of fresh and frozen ground beef products were recalled *(14)*.

Preharvest research has shown that beef cattle are intestinally colonized at higher prevalence levels than previously thought and that slaughter-ready cattle in feedlots might have higher contamination and colonization levels in the oral cavity and on their hides than in their feces *(16, 24)*. EC O157 feedlot prevalence levels can be affected by the entry of previously colonized calves *(27)*. Calves from cow-calf operations can go directly to feedlots or be enrolled in preconditioning programs prior to entry into the feedlot *(46)*. Few studies have evaluated EC O157 prevalence levels in weaned calves prior to their entry into feedlots. Laegreid et al. *(27)* found that weaned range calves in 13 of 15 herds (87%) from five states were shedding EC O157 prior to entry into the feedlots. Within the positive herds, prevalence ranged from 1.7 to 20% (mean = 7.4%). In a study of Kansas cow-calf farms ($n = 10$), EC O157 was isolated from cattle on all 10 farms. Among calves, the prevalence of EC O157 was 1.4% ($n = 804$) *(39)*. Both studies used immunomagnetic separation and a large fecal sample (10 g) in their culture protocol, methods that are more sensitive than conventional enrichment or direct plating detection techniques *(9, 16, 26, 28, 42)*.

Commingling of calves provides a mechanism for transfer of microbial flora between animals (horizontal transmission) and might contribute to episodic or epidemic fecal shedding *(6, 27)*. Several studies suggested that newly arrived cattle at feedlots and those ''on feed'' for the short-

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est amount of time had higher prevalence rates of EC O157 than animals acclimated to the feedlot environment *(20, 47, 48).* This observation has been attributed to changes in the gastrointestinal flora that might occur secondary to dietary stress associated with adjustment to feedlot rations *(20, 27, 38).* In contrast, Smith et al. *(41),* in a study of midwestern feedlots, found that prevalence of EC O157 was not correlated with the number of days on feed.

The feedlot industry is concentrated in the midwestern and southwestern United States; however, steers and heifers originate from farms and ranches throughout the United States *(46).* These feedlot steers and heifers make up a large portion of the animals used for meat in U.S. beef markets *(46).* Louisiana is predominantly a cow-calf production state *(44).* The Louisiana Calf to Carcass (CTC) project is an annual program designed by the Louisiana State University Agricultural Center to help cattle producers optimize management and maximize profits through a preconditioning program *(12).* In conjunction with the Louisiana CTC program, a census sampling of calves enrolled in the 2001 program was performed. Fecal samples were taken at the beginning and at the end of the 45-day preconditioning period.

The objectives of this study were to estimate the point prevalence of EC O157 fecal shedding in the cohort of calves and to determine whether changes in fecal prevalence levels occurred following the 45-day preconditioning (feeding) period. EC O157 isolates obtained in the study were confirmed and characterized. Pulsed-field gel electrophoresis (PFGE) was performed to evaluate genetic diversity of the isolates and to document horizontal transmission of EC O157 subtypes.

MATERIALS AND METHODS

CTC program and sampling protocol. Calves were enrolled that met CTC program criteria *(12).* To participate, producers were required to consign a minimum of three calves. Producers delivered the consigned calves on 6 September 2001 to one of three preconditioning sites (PC sites), designated A, B, and C.

On arrival, calves were weighed, vaccinated (BRD complex and eight-way clostridial vaccines), given a metaphylactic antibiotic injection (Micotil, Elanco, Indianapolis, Ind.), and treated for internal and external parasites. Calves were commingled and pastured in one area (PC site B) or in two or more adjacent pastures (PC sites A and C). Animals were revaccinated on 24 September 2001. Calves were maintained on Alicia and Coastal Bermuda grass pastures and fed Alicia and Bermuda grass hay free choice, concentrate, minerals, and water. The concentrate, a medicated transition ration, was fed at a daily rate of 1.5% body weight and contained 12% crude protein, 15% crude fat, and 26% crude fiber. The concentrate was supplemented with oxytetracycline (200 g/ton) and is labeled for feeding weaned beef cattle on pasture or in the feedlot to prevent and treat shipping fever complex. Following the 45-day preconditioning period, the calves were shipped to a feedlot in the midwestern United States.

To determine the prevalence of EC O157, fecal samples ($n = 408$) were taken on arrival from the farms of origin (designated time 1, T1) and again ($n = 453$) following the 45-day preconditioning period (time 2, T2). Approximately 20 g of feces was obtained per rectum with the use of a new glove for each animal. Fecal samples were maintained at ambient temperature and placed in media within 24 h.

Culture methods. Immunomagnetic separation culture techniques were used that have demonstrated greater sensitivity than conventional enrichment and direct plating techniques *(9, 16, 26, 30, 42).* The culture techniques have been described previously *(24).*

Briefly, 10 g of fresh bovine feces was incubated at 37°C for 6 h in 90 ml of gram-negative broth (Difco, Becton Dickinson, Sparks, Md.) supplemented with cefsulodin (10 mg/liter; Sigma, St. Louis, Mo.), vancomycin (8 mg/ml; Sigma), and cefixime (0.05 mg/ml; Lederle, Pearl River, N.Y.). Immunomagnetic separation was performed on a 1-ml aliquot with the use of anti–*E. coli* O157 Dynabeads (Dynal Biotech Inc., Lake Success, N.Y.).

The immunomagnetic bead and bacteria complex (50 μl) was spread plated on sorbitol MacConkey agar (SMAC; Difco) containing cefixime (0.05 mg/liter; Dynal Biotech) and potassium tellurite (ctSMAC, 2.5 mg/liter; Dynal Biotech) and incubated at 37°C for 18 to 24 h. A maximum of three colonies with typical Shiga-toxigenic *E. coli* (STEC) O157:H7 phenotypic characteristics were selected as suspects and placed into 5 ml of MacConkey broth and 2 ml of Trypticase soy broth for 18 to 24 h at 37°C.

Serotype confirmation by enzyme-linked immunosorbent assay (ELISA). Aliquots of broth culture were heat killed at 100°C for 5 min and tested by indirect immunoassay for reactivity with anti-H7 monoclonal antibody (MAb) 2B7 *(21)* and anti-O157 MAb 13B3 *(49),* as previously described *(24).*

Isolates reactive with MAb 13B3 (O157-positive ELISA) were inoculated in Trypticase soy broth and evaluated for motility by phase-contrast microscopy. Isolates with the correct phenotype (1 to 2 mm, sorbitol-negative colonies) on ctSMAC and that fermented lactose in MacConkey broth (yellow color change), reacted with anti-O157 MAb 13B3, and reacted with anti-H7 MAb 2B7 or were nonmotile (NM) were considered to be *E. coli* O157:H7 or *E. coli* O157:NM, respectively. A single isolate from each positive sample was archived in a brain heart infusion broth–glycerol suspension at −80°C for future characterization.

Characterization by PCR. Isolates were characterized by PCR for rfb_{O157} and $fliC_{H7}$ and the putative virulence factors stx_1, stx_2, *eaeA,* and *hlyA.* Somatic (O157), flagellar (H7), and virulence factor gene sequences were amplified with the use of previously published primer pair sequences *(15, 33).* Duplex reactions were run for stx_1 and stx_2, as well as for *eaeA* and rfb_{O157}. Uniplex PCR reactions were run for *hlyA* and $fliC_{H7}$. PCR cycling conditions were as previously described *(15, 33).*

Amplified gene products were electrophoresed on a 2% agarose gel and stained. Gel images were captured digitally, photographed, and scored. Clear bands of the correct size for rfb_{O157} (259 bp), $fliC_{H7}$ (625 bp), stx_1 (180 bp), stx_2 (255 bp), *eaeA* (384 bp), and *hlyA* (534 bp) that were consistent with the positive control were considered positive PCR reactions.

Molecular subtyping of isolates. All EC O157 isolates were subtyped by enzyme restriction and PFGE as previously described *(27).* For restriction enzyme digestion, *Xba*I was selected. However, electrophoresis revealed that enzyme digestion did not occur or was incomplete on three separate attempts. A second enzyme, *Spe*I (New England Biolabs, Beverly, Mass.) was used to digest isolate DNA. Following PFGE separation of *Spe*I-restricted DNA, gels were stained. Genetic relatedness of epidemiologically related isolates within herds was determined according to the criteria of Tenover et al. *(43).* Strict criteria for isolates in epidemiologically unlinked herds (between herds) were used; isolates differing by

TABLE 1. Escherichia coli *O157:H7 (EC O157) prevalence proportions and confidence intervals (95% CI) in the 2001 Louisiana Calf to Carcass cohort at arrival (T1) and after preconditioning (T2)*

Site	Prevalence[a]	95% CI[b]
Herd-level prevalence (T1)		
A	4/13 = 31.0%	9–61
B	0/9 = 0%	0–34
C	1/7 = 14.3%	0–53
All	5/29 = 17.2%	6–36
Animal-level prevalence (T1)		
A	8/177 = 4.5%	2–9
B	0/66 = 0%	0–5
C	2/165 = 1.2%	0–4
All	10/408 = 2.5% A	1–5
Animal-level prevalence (T2)		
All	0/453 = 0% A	0–1
Animal-level prevalence and herd (site-herd)		
A-9	1/45 = 2.2%	0–9
A-12	2/35 = 5.7%	1–19
A-3	4/10 = 40.0%	12–74
A-6	1/16 = 6.3%	0–30
C-1	2/6 = 33.3%	4–78

[a] Proportions followed by a letter are significantly different by Fisher's exact test ($P < 0.01$).

[b] Exact 95% confidence interval for proportion.

more than two bands were considered separate subtypes *(2, 3, 22, 42).*

Statistical analysis. Prevalence estimates with 95% confidence intervals (CIs) were calculated as the number of positive samples divided by the total number of samples (PEPI 4.01, Sagebrush Press, Las Vegas, Nev.). Fisher's exact statistics were used to estimate CIs for null values. Prevalence proportions were compared statistically at the 95% level of confidence by exact methods.

RESULTS

CTC cohort enrollment. Twenty-nine cow-calf farms enrolled calves in the 2001 CTC program. The PC sites were located in southeastern (Louisiana State University), southwestern (McNeese State University), and northern Louisiana (Louisiana Tech University). Calves originated from 21 of Louisiana's 64 (31.8%) parishes. The number of animals enrolled from individual herds at each preconditioning location was highly variable (mean = 14.1, SD = 16.2). Four herds (n = 47 calves) were sampled at T2 that were not sampled initially. These calves participated in the program but were preconditioned at home. At T1, 408 calves were sampled. Following the preconditioning period, 453 calves were sampled. Of the 408 calves sampled initially, 406 (99.5%) were sampled at T2.

Prevalence proportions. *E. coli* O157 isolates were obtained from sampling at T1. Fecal point prevalence was 2.5%. No isolates were obtained at T2. Table 1 reports the herd-level prevalence proportions, the animal-level prevalence proportions, and the prevalence proportions from positive herds at each preconditioning site at T1. Overall point estimates (prevalence proportions) for fecal shedding of EC O157 at T1 and T2 are reported and compared statistically with exact methods (Table 1).

Composite characterization and PFGE. Isolates were considered EC O157 on the basis of ELISA results and PCR amplification of rfb_{O157} and $fliC_{H7}$ genes. Isolates were further classified as STEC O157:H7 if PCR reactions for stx_1 or stx_2 or both were positive. We detected EC O157 isolates from multiple herds that were *stx*-deficient. Genetic characterization data and PFGE subtype classification for the isolates obtained during the CTC study are reported in Table 2.

DISCUSSION

Louisiana's calf production in 2001 was an estimated 405,000 calves, approximately 1% of the U.S. total *(44).* Our goal was to obtain a cross-sectional sample of weaned Louisiana beef calves destined for the feedlot to estimate the prevalence of EC O157:H7 fecal shedding. EC O157 has not been reported in Louisiana beef cattle. On the basis of previous studies, we expected to find (i) low animal-level prevalence of EC O157:H7 fecal shedding in weaned calves at T1, (ii) higher prevalence in the cohort following commingling of positive and negative animals placed on the transition ration, and (iii) horizontal transmission of unique EC O157:H7 subtypes at T2 in initially (T1) negative calves.

TABLE 2. *Composite PCR and pulsed-field gel electrophoresis results for* Escherichia coli *O157 (EC O157) isolates from the 2001 Louisiana Calf to Carcass study*

Site-herd	n	rfb_{O157}	$fliC_{H7}$	stx_1	stx_2	*eaeA*	*hlyA*	PFGE type[a]
A-9	1	+	+	−	−	+	+	A
A-12	2	+	+	−	−	+	+	B[b]
A-3	4	+	+	−	−	+	+	C[c]
A-6	1	+	+	−	+	+	+	D
C-1	2	+	+	−	+	+	+	E, F[d]

[a] PFGE subtype classification from Tenover et al. *(43)* for within-herd classification. For isolates between herds, subtype was based on a difference of two or more bands.

[b] One of the two isolates from A-12 was not available for PFGE (isolate died).

[c] Isolates considered indistinguishable.

[d] Isolates from C-1 considered possibly related.

Sampling for the CTC study was not statistically based. Each producer chose the number of caves to be enrolled from their farms. This nonrandomized, nonsystematic enrollment scheme likely resulted in selection bias in our results. Selection bias is defined as distortions resulting from procedures used to select subjects and from factors that influence voluntary participation in studies *(37)*. Selection bias that affects the validity and generalizability of our cross-sectional point prevalence estimates was inherent because sampling was based on convenience.

Prevalence proportions and 95% CIs are shown in Table 1. Five of the 29 herds enrolled in the CTC program were found to be shedding EC O157. Our findings are consistent with those of the previous studies on cow-calf farms and in weaned calves from other regions in the United States that demonstrated a low animal-level prevalence of EC O157 *(27, 39)*. The animal-level prevalence was 2.5% (CI, 1.2–4.5). Herd-level prevalence (17.2%) was lower than that of the two previously mentioned studies *(27, 39)*. The small numbers of calves that were enrolled from some herds make interpretation of herd-level prevalence estimates problematic. We feel that EC O157 would have been found in more herds if a statistically based sampling criterion could have been used.

Our results showed a statistically significant ($P < 0.01$) difference in the proportion of calves that were shedding EC O157 at time T1 (2.5%) compared with time T2 (0%) (Table 1). Colonization of cattle by EC O157 appears to occur quickly, although fecal shedding is transient (2 to 3 weeks to months) *(5, 6, 11)*. Furthermore, calves are susceptible to horizontal or calf-to-calf transmission at very low doses of the bacterium. Besser et al. *(6)* suggested that low infectious doses provide a high risk of infection for susceptible calves. Rapid transmission between susceptible calves could explain temporal increases in fecal shedding when a susceptible cohort is exposed to EC O157 *(6)*. Thus, we expected to see an increase in fecal shedding following the 45-day feeding period rather than the absence of EC O157 fecal shedding.

Biologically plausible hypotheses that account for the absence of fecal shedding at T2 include (i) climate changes during the 45-day feeding period, (ii) antibiotic (oxytetracycline) in the ration, or (iii) horizontal transmission and fecal shedding that ended prior to T2.

Seasonal changes in fecal shedding have been shown in cattle, similar to seasonal changes observed in human disease incidence *(4, 8, 19, 28)*. In fact, Hancock et al. *(18)* state that the ''harmony'' that exists between seasonal increases in STEC O157:H7 fecal shedding in cattle and increases in human disease incidence is evidence of causation. Concurrent studies in Louisiana dairy cattle detected a limited amount of EC O157 fecal shedding in October 2001. It is plausible that by mid-October (T2), seasonal factors or other unknown factors that coincide with colder months were limiting EC O157 colonization or fecal shedding or both. The statewide mean temperature in October 2001 (64.7°F) was cooler than normal for Louisiana *(45)*.

Oxytetracycline was a component in the transition ration at 200 g/ton. The ration is labeled for the prevention and early treatment of shipping fever (primarily gram-positive respiratory pathogens). However, oxytetracycline is a broad-spectrum bacteriostatic drug *(34)*. Tetracycline resistance has been reported in EC O157. Isolates were susceptible to tetracycline and oxytetracycline but intermediately resistant or resistant to chlortetracycline (data not shown) *(29, 40)*. Parenteral administration of tetracycline to calves has been reported to have no effect on EC O157 fecal shedding *(35)*.

Studies have shown that growth-promoting antibiotic concentrations vary widely in the gastrointestinal tract *(25)*. Pharmacokinetic properties of oxytetracycline make determination of intralumenal concentrations difficult to estimate. Bioavailability following oral administration is reported to be 60 to 80%; however, the presence of food in the digestive tract can reduce the amount of tetracycline absorbed by 50% or more *(34)*. Further complicating matters, oxytetracycline might be progressively inactivated as it passes through the intestinal tract because of chelation with ingesta or fecal material. Conclusions regarding the effect of antibiotic supplementation with respect to the absence of EC O157 fecal shedding cannot be made.

Antimicrobials in animal feeds are reported to enhance animal growth by 4 to 5% *(25)*. Concerns by consumers and health officials regarding antibiotic resistance of foodborne pathogens and the role of antibiotic-supplemented animal feeds are warranted *(10)*. An additional concern related to STEC is the induction of *stx*-converting phages when bacteria are exposed to subinhibitory concentrations of antibiotics. In vivo induction of phages from lysogenic STEC could increase free *stx*-converting phage and subsequently cause the spread of stx and creation of new STEC pathotypes *(25)*.

A third plausible hypothesis that might explain the absence of fecal shedding at T2 is that transmission, colonization, and fecal shedding ended prior to sampling. Early transmission and the cessation of fecal shedding would not have been detected because sampling did not occur throughout the feeding period.

An unexpected and unusual finding in our study was the isolation of *stx*-negative EC O157 (Table 2), which possessed other virulence factors (*eaeA* and *hlyA*) and flagellar gene sequences ($fliC_{H7}$) commonly found in the EC O157 clone associated with human illness. The *stx*-negative EC O157 isolates were classified as three distinct PFGE subtypes (A, B, and C; Table 2) consistent with their herd of origin. The significance of the *stx*-deficient EC O157:H7 isolates is unclear. Non–Shiga-toxigenic EC O157 has been recognized in cattle. Itoh et al. *(23)* proposed that *stx*-deficient EC O157 might exist widely in cattle. Sequence variation (insertion sequence) of *stx* has been reported to cause *E. coli* isolates to appear *stx*-deficient with the use of certain primer pairs *(32)*.

Multiple isolates from one herd (A-3, Table 2) had indistinguishable PFGE profiles. On the basis of the criterion of Tenover et al. *(43)*, the EC O157 isolates from herd C-1 were considered possibly related. We observed unique subtypes of EC O157 in epidemiologically unlinked herds, although subtypes did vary in one herd. Laegreid et al. *(27)*,

in their study of weaned calves, stated that most herds had one or two closely related patterns, but five herds in their study had EC O157 isolates with two distinct patterns. We expected, but were unable to demonstrate, horizontal transmission of distinct PFGE subtypes. Conclusions regarding the number and diversity of subtypes in our study are limited.

We observed that the prevalence proportion of EC O157 in weaned Louisiana beef cattle was consistent with that of previous studies in weaned range calves. The presence of *stx*-deficient EC O157:H7 was an unexpected finding in this study. We expected, but did not observe, increased fecal shedding or horizontal transmission of EC O157 subtypes at T2. The absence of fecal shedding following the 45-day feeding period might be attributable to seasonal influences, inhibitory concentrations of oxytetracycline in the transition ration, or transient colonization that ended prior to T2. EC O157 is apparently widely dispersed at low prevalence in weaned calves from U.S. cow-calf herds.

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